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(57) Abstract

An immunological oral tolerance–inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$–glycoprotein–1(β_2GP–1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

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A COMPOSITION FOR THE PREVENTION AND/OR TREATMENT OF ATHEROSCLEROSIS

Technical Field

The present invention relates to an immunological oral tolerance-inducing composition for the prevention and/or treatment of atherosclerosis.

Background Art

Atherosclerosis and its clinical sequelae represent one of the most hazardous insults on Western society in terms of morbidity and mortality. Hence, it is not surprising that immense efforts have been put forward to investigate its etiopathogenesis aiming to provide means of slowing or halting its early indolent progression. Although no definite answers exist it is becoming apparent that not one individual mechanism is solely responsible for the evolvement of the atherosclerotic plaque as was formerly presumed with regard to dysregulation of the lipid balance.

Clinicians use the expression atherosclerosis to define a histopathological process which results in occlusion of arteries in different parts of the body. The occlusion is caused by the accumulation of different cells of various origins which are gradually being filled with large amounts of fat from the circulation. The fat which mainly consists of 'bad' cholesterol low density lipoprotein (LDL), is the principal material which deposits in the vessel walls. In the later stages of atherosclerosis, the lipid filled zones begin to accumulate calcium. The deposition of calcium renders the arteries stiffer and less flexible, thereby causing their 'stony' appearance, an appearance which has led to the well-known expression of "sclerosis of the arteries" (arteriosclerosis). When the involved arteries block the blood flow to the heart, a person is afflicted with a 'heart attack'; when the brain arteries occlude, the person experiences a stroke. When arteries to the limbs narrow, the result is severe pain, decreased physical mobility and possibly the need for an amputation.

It is clear that the progression of the above-mentioned diseases is non-detectable for many years and only manifests themselves at advanced stages. When these manifestations become detectable it is much more difficult to offer a remedy, due to the advanced stages of the disease.

As mentioned above, atherosclerosis is the result of the deposit of fat in the walls of blood vessels, thereby creating a layer of atherosclerotic plaque, said layer

clogs the flow of blood to vital organs, thereby leading to cerebrovascular accidents, myocardial infarctions or peripheral blood vessel diseases. Known risk factors for rapid progression of the condition include high blood pressure, diabetes, overweight, smoking and the lack of physical exercise.

In recent studies it appears that infectious factors such as Cytomegalo virus (CMV), Epstein-Bar (EBV) and Chlamydia pneumonia may also be involved in the progression of atherosclerosis. The above has been demonstrated by linking atherosclerosis with gingivitis, in addition to the correlation between vessel obstruction and restenosis and the titers of autoantibodies to anti-CMV antigens, or finding infectious particles in the atherosclerotic plaque.

In the last decade a body of evidence has been accumulated to support the theory that atherosclerosis has a significant infectious-autoimmune component.

In autoimmune diseases the immune system attacks our body components (autoantigens), in addition to attacking external invaders. The autoimmune diseases are classified as autoantibody mediated or cell mediated diseases. Typical autoantibody mediated autoimmune diseases are myasthenia gravis and idiopathic thrombocytopenic purpura (ITP), while typical cell mediated diseases are Hashimoto's thyroiditis and Diabetes type I.

Involvement of the immune network in atherosclerosis

The recognition that immune mediated processes prevail within atherosclerotic lesions stemmed from the consistent observation of lymphocytes and macrophages in the earliest stages, namely the fatty streaks. These lymphocytes which include a predominant population of CD4+ cells (the remainder being CD8+cells) were found to be more abundant over macrophages in early lesions, as compared with the more advanced lesions, in which this ratio tends to reverse. These findings posed questions as to whether they reflect a primary immune sensitization to a possible antigen or alternatively stand as a mere epiphenomenon of a previously induced local tissue damage. Regardless of the factors responsible for the recruitment of these inflammatory cells to the early plaque they seem to exhibit an activated state manifested by concomitant expression of MHC class II HLA-DR and interleukin (IL) receptor as well as leukocyte common antigen (CD45R0) and the very late antigen 1 (VLA-1) integrin.

The on-going inflammatory reaction in the early stages of the atherosclerotic

lesion may either be the primary initiating event leading to the production of various cytokines by the local cells (i.e endothelial cells, macrophages, smooth muscle cells and inflammatory cells), or it may be that this reaction is a form of the body's defense immune system towards the hazardous process. The cytokines which have been shown to be upregulated by the resident cells include TNF-α, IL-1, IL-2, IL-6, IL-8, IFN-γ and monocyte chemoattractant peptide-1 (MCP-1). Platelet derived growth factor (PDGF) which is expressed by all cellular constituents within atherosclerotic plaques have also been shown to be overexpressed, thus possibly intensifying the preexisting inflammatory reaction by a co-stimulatory support in the form of a mitogenic and chemotactic factor. Very recently, Uyemura K, Demer LL, Castle SC et al. Cross regulatory roles of IL-12 and IL-10 in atherosclerosis. J Clin Invest 1996 97; 2130-2138 have elucidated type 1 T-cell cytokine pattern in human atherosclerotic lesions exemplified by a strong expression of IFN-γ but not IL-4 mRNA in comparison with normal arteries. Furthermore, IL-12 - a T-cell growth factor produced primarily by activated moncytes and a selective inducer of Th1 cytokine pattern, was found to be overexpressed within lesions as manifested by the abundance of its major heterodimer form p70 and p40 (its dominant inducible protein) mRNA.

Similar to the strong evidence for the dominance of the cellular immune system within the atherosclerotic plaque, there is also ample data supporting the involvement of the local humoral immune system. Thus, deposition of immunoglobulins and complement components have been shown in the plaques in addition to the enhanced expression of the C3b and C3Bi receptors in resident macrophages.

Valuable clues with regard to the contribution of immune mediated inflammation to the progression of atherosclerosis comes from animal models. Hence, it seems that immunocompromised mice (class I MHC deficient) tend to develop accelerated atherosclerosis as compared with immune competent mice. Additionally, treatment of C57BL/6 mice (Emeson EE, Shen ML. Accelerated atherosclerosis in hyperlipidemic C57BL/6 mice treated with cyclosporine A. Am J Pathol 1993; 142: 1906-1915) and New-Zealand White rabbits (Roselaar SE, Schonfeld G, Daugherty A. Enhanced development of atherosclerosis in cholesterol fed rabbits by suppression of cell mediated immunity. J Clin Invest 1995; 96:

1389-1394) with cyclosporine A, which is- a potent suppressor of IL-2 transcription resulted in a significantly enhanced atherosclerosis under "normal" lipoprotein "burdeon". These latter studies may provide insight into the possible roles of the immune system as engaged in counteracting the self-perpetuating inflammatory process within the atherosclerotic plaque.

Atherosclerosis is not a classical autoimmune disease, although some of its manifestations such as the production of the plaque which obstruct the blood vessels may be related to immune system effects. In classical autoimmune disease, one can also define very clearly the autoanigen attacked by the immune system, one can define the part of the immune system which attacks the autoantigen (autoantibody or cells) which belong to the immune system and are named lymphocytes. Above all one can show that by passive transfer of these components of the immune system the disease can be induced in healthy animals, or in the case of humans the disease may be transferred from a sick pregnant mother to her offspring. Many of the above are not prevailing in atherosclerosis. In addition, the disease definitely has common risk factors such as hypertension, diabetes, lack of physical activity, smoking and others, the disease affects elderly people and has a different genetic preponderance than in classical autoimmune diseases.

The process of inducing oral tolerance has been known since the beginning of the century for reducing allergic reactions. The allergic patient was fed with a low dose of the known allergen and the body's immune tolerance was restored and no allergic reaction occurred.

Oral tolerance in autoimmune diseases

In autoimmune diseases oral tolerance is a term denoted to describe blunting the immune response in an autoimmune disease. The tolarization produced by feeding an animal with the 'incriminated' antigen, can abrogate the development of the disease due to a 'paralyzing' effect on the immune response. The present inventors have recently shown that oral feeding with human β2GPI prior to immunization with this molecule, resulted in amelioration of the anti-phospholipid (APS)-equivalent syndrome.

Oral tolerance has been applied in the realm of autoimmune diseases, in which an immune reaction was carried out against the autoantigen and there existed a need for restoring the immune system's tolerance to the autoantigen.

This was carried out by feeding a subject with low doses of the autoantigen. So far, several animal models have been reported in which oral tolerance has been restored, including preventing the development of experimental allergic encephalomyelitis (EAE), which is the equivalent of multiple sclerosis (MS), by feeding a subject with a protein from the nerve membrane called myelin basic protein (MBP), in addition to preventing adjuvant arthritis and collagen arthritis, by feeding a subject with collagen and HSP-65, respectively. A Boston based company called Autoimmune has carried out several human experiments for preventing diabetes, multiple sclerosis, rheumatoid arthritis and uveitis. The results of the human experiments have been less impressive than the non-human ones, however there has been a success with the prevention of arthritis.

Antigenic Components

A. Oxidized low density lipoprotein

LDL is a complex of large molecular weight proteins, including apolipoprotein B, neutral and polar lipids and lipophilic antioxidants (vitamin E and β carotene). The oxidation-modification of LDL with the resultant formation of neoepitopes within the native molecule (within the Apo B domain) leads to its recognition by the scavenger receptor on the macrophages. It is possible that all cellular components in the atherosclerotic plaque (i.e. endothelial cells, macrophages, smooth muscle cells, lymphocytes) are capable of enhancing lipid peroxidation with the production of varying degrees of LDL oxidation, yet the relative contribution of each, has not been determined. Regardless of the precise roles of these cells it appears that the balance between the oxidant and antioxidant forces at the level of the vessel walls determines the extent of exposure to Ox LDL with the subsequent deleterious effects. Ox LDL has active derivatives such as lysophosphatidylcholine (LPC) and, despite having a smaller molecular weight, still retains some of its biological activities.

Lysophosphatidylcholine (LPC) is expressed in human atherosclerotic plaques. It is an active biological substance that can induce the first steps of atherogenesis. Indeed it is even more potent than Ox LDL.

The overall *in-vivo* and *in-vitro* influence of Ox LDL and its byproducts on the development of the plaque are well beyond the scope of the present invention,

however it is important to state the known relationship between Ox LDL and the immune system.

It is known that Ox LDL is chemotactic for T-cells and monocytes. Ox LDL and its byproducts are also known to induce the expression of factors such as monocyte chemotactic factor 1, secretion of colony stimulating factor and platelet activating properties, all of which are potent growth stimulants.

The active involvement of the cellular immune response in atherosclerosis has recently been substantiated by Stemme S, Faber B, Holm J. T lymphocytes from human atherosclerotic plaques recognize oxidiezed low density lipoprotein. Proc Natl Acad Sci USA 1995; 92: 3893-97, who isolated CD4+ within plaques clones responding to Ox LDL as stimuli. The clones corresponding to Ox LDL (4 out of 27) produced principally interferon-γ rather than IL-4. It remains to be seen whether the above T-cell clones represent mere contact with the cellular immune system with the inciting strong immunogen (Ox LDL) or that this reaction provides means of combating the apparently indolent atherosclerotic process.

The data regarding the involvement of the humoral mechanisms and their meaning are much more controversial. Several reports have associated increased levels of antibodies to Ox LDL with the progression of atherosclerosis (expressed by the degree of carotid stenosis, severity of peripheral vascular disease etc.). However, these data were not reproduced by other scientists, perhaps due to the lack of standardization with regard to the assays used for determining antibodies to LDL. In any case there seems to be a consensus as to the presence of Ox LDL antibodies in the form of immune complexes within atherosclerotic plaque, although the true significance of this finding has not been established.

Antibodies to Ox LDL have been hypothesized as playing an active role in lipoprotein metabolism. Thus, it is known that immune complexes of Ox LDL and its corresponding antibodies are taken up more efficiently by macrophages in suspension as compared with Ox LDL. No conclusions can be drawn from this consistent finding on the pathogenesis of atherosclerosis since the question of whether the accelerated uptake of Ox LDL by the macrophages is beneficial or deleterious has not yet been resolved.

Important data as to the significance of the humoral immune system in atherogenesis comes from animal models. It has been found that

hyperimmunization of LDL-receptor deficient rabbits with homologous oxidized LDL, resulted in the production of high levels of anti-Ox LDL antibodies and was associated with a significant reduction in the extent of atherosclerotic lesions as compared with a control group hyperimmunized with phopsphate-buffered saline (PBS). A decrease in plaque formation has also been accomplished by immunization of rabbits with cholesterol rich liposomes with the concomitant production of anti-cholesterol antibodies, yet this effect was accompanied by a 35% reduction in very low density lipoprotein cholesterol levels. The present inventors have shown that in apoE knockout mice, repeated immunizations with homologous Ox LDL results in production of anti-Ox LDL antibodies and in reduced atherosclerosis.

B. Heat Shock protein (HSP) 60/65

An additional major antigenic component for the initiation and perpetuation of the inflammatory lesion culminating in enhanced atherosclerosis is the 60 Kd heat shock protein. This mitochondrial protein is a member of the HSP family which constitutes about 24 proteins displaying high degree of sequence homologies between different species. These proteins, as their name implies are upregulated in response to various stressful insults including exposure to free radicals, heat, mechanical shear stress, infections, cytokines ect. The teleological importance of the HSP stems from their protective roles against unfolding of cellular proteins precipitated by stressful stimuli. This role has led to their designation as molecular 'chaperones'. However, these apparently favorable effects of HSP's can be a double-edged sword, since their overexpression may, under certain conditions promote an autoimmune reaction with resultant tissue damage. The mechanisms responsible for the HSP immune mediated damage are not completely understood and it is presumed that cryptic neoepitopes are exposed to the immune system which no longer consider them as 'self' following their upregulation. Alternatively, it was suggested that cross reaction exists between 'foreign' HSP and self HSP introduced following infections which act to trigger an immune attack against self structures.

HSP 60 is a distinct protein which constitutes a separate "class" within the above-mentioned HSP family, together with HSP 65, providing a sequestered environment for folding a subset of proteins in-vivo.

There exists a similarity between mammalian HSP60 and bacterial HSP65, which allows for cross recognition by immune effectors of the host.

Support for the involvement of HSP in autoimmunity is provided by studies documenting enhanced autoantibody as well as cellular response to HSP 60/65 in several autoimmune diseases.

The link between HSP 65 and atherosclerosis was initially raised following a pioneering work conducted by Georg Wick's group in the early 1990s (Xu Q, Dietrich H, Steiner HJ & al. Induction of arteriosclerosis in normocholesterolemic mice rabbits by immunization with heat shock protein 65. Arteriosclerosis Thrombosis 1992; 12: 789-799). They found that rabbits immunized with different antigens developed atherosclerosis, provided the preparation used for immunization contained complete Freund's adjuvant (CFA). Since the major constituent of CFA is heat .killed mycobacterium tuberculosis the principal component of which is the HSP-65, they reasoned that the immune response towards this component led to the development of atherosclerosis. This hypothesis was confirmed when the animals were subsequently immunized merely with HSP-65 and exhibited pronounced atherosclerosis. It should be emphasized that no difference was noted between the groups with regard to the lipoprotein profile. Additional work conducted later by Wick's group revealed that T cells extracted from lesions of the rabbits induced to develop atherosclerosis were found to overexpress HSP-65 in comparison with peripheral blood from the respective animals thus attesting for a localized and restricted immune reaction in the vicinity of the stressed arterial vessel. The present inventors have reinforced Wick's finding, by showing that HSP-65 (or Mycobacterium tuberculosis) immunization of naive mice resulted in accelerated fatty streak formation.

These findings were further substantiated when involvement of humoral immune mechanisms in response to HSP-65 were observed in patients inflicted with atherosclerosis. Hence, a correlation has been established between high levels of anti-HSP65 antibodies and the extent of sonographically estimated carotid narrowing in a screen of healthy individuals.

These findings were recently reinforced by *in-vitro* assays designed to assess the influence of HSP-60/65 on cultured endothelial cells. Accordingly, it has been shown that incubation of endothelial cells with HSP65 induced their

adhesiveness to monocytes and granulocytes which was concentration and time dependent. Furthermore, it has been demonstrated that this effect is mediated by overexpression of endothelial cells E-selectin (C62E), vascular cell adhesion molecule-1 (CD106) and intracellular adhesion molecule-1 (CD54).

Phospholipids and β2GPI as autoantigen components in atherosclerosis.

The present inventors have found that the production of anticardiolipin in LDL-receptor knockout mice by immunization with human anticardiolipin antibodies (aCL) results in accelerated early atherosclerosis. This observation is consistent with circumstatial data that has accumulated with regard to the possible proatherogenic role of anticardiolipin antibodies in atherosclerosis.

β2GPI is a molecule that was proved as the target of aCL from patients with autoimmune diseases. Anti-β2GPI antibodies have been shown to result in experimental APS and to activate endothelial cells and platelets. The present inventors have shown that trasgenic LDL-RD deficient mice immunized with β2GPI develop respective antibodies and that early fatty streak formation is enhanced, accompanied by local accumulation of CD4+ cells. Thus, β2GPI may serve as an autoantigenic target of the autoimmune response in atherosclerosis.

In both animal and human disease models a correlation has been found between the titer autoantibodies to the autoantigens HSP 60-65 and Ox LDL and the level of atherosclerosis. The Ox LDL is produced in the body by the oxidation of the native LDL and is considered the noxious factor of atherosclerosis. In response to said toxin, anti-Ox LDL antibodies are produced. The β2GP-I is a natural protein in human blood which plays a role in the coagulation process and HSP65 is the result of various stress stimuli.

In experiments carried out with mice having a predisposition for the development of atherosclerosis (LDL-R knock-out mice), a thirty percent reduction was demonstrated in the atherosclerosis condition by feeding the mice with low doses of Ox LDL.

Furthermore, the fact that the immune system is involved in the induction of atherosclerosis, as well as the clearcut involvement of the immune system (lymphocytes and autoantibodies), points to the ability to manipulate the disease by inducing oral tolerance with autoantigens, e.g., Ox LDL, HSP60/65 and β_2GPI.

U.S. Patent No. 5,348,945 discloses a method of combating mortality in a cell or tissue under stress. The method comprises contacting heat shock protein 70 (HSP70) to the cell or tissue in an amount effective to enhance the survival of that cell or tissue. The method may be employed in the combating of atherosclerosis, restenosis after angioplasty and nerve damage in human or animal subjects in need of such treatment. A pharmaceutical composition comprising a therapeutically effective amount of HSP70 in a pharmaceutically acceptable formulation is also disclosed.

Although HSP70 and HSP60 belong to a family of about 24 highly conserved heat shock proteins, they represent two entirely distinct characteristics. Their mechanism, for example, do not appear to act in concert in governing the protection from stressful stimuli.

HSP70 was initially patented because of its pronounced induction during heat exposure and other stressful insults such as ischemic preconditioning. Indeed the overexpression of HSP70 in transgenic animals is associated with protection from stressful hazards.

Therefore, U.S. Patent No. 5,348,945 does not teach or suggest the subject matter of the present invention.

Disclosure of the Invention

Thus, according to the present invention there is now provided an immunological oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In an preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of a heart attack, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In another preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of angioplasty-restenosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a further preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of stroke, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In even further preferred embodiments of the present invention there is provided an immunological oral tolerance-inducing composition wherein said active component is a modified low-density lipoprotein, or wherein said active component is oxidized low-density lipoprotein (Ox LDL), or wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL), or wherein said active component is heat shock protein 60/65 (HSP 60/65), or wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

The present invention provides an immunological oral tolerance-inducing composition, wherein said active derivative is lysophosphatidyl choline (LPC).

The present invention also provides an immunological oral tolerance-inducing composition, wherein said LDL is malondialdehyde LDL (MDA-LDL).

In another aspect of the present invention there is provided a method for prevention and/or treatment of atherosclerosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and

mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a preferred embodiment there is provided a method for prevention and/or treatment of a heart attack in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a further preferred embodiment there is provided a method for prevention and/or treatment of angioplasty-restenosis following angioplasty in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In an even further preferred embodiment there is provided a method for prevention and/or treatment of stroke in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In further embodiments of the present invention there is provided a method for prevention and/or treatment of atherosclerosis in a subject, wherein said active component is a modified low-density lipoprotein, or wherein said active component is oxidized low-density lipoprotein (Ox LDL), or wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL), or wherein said active component is heat shock protein 60/65 (HSP 60/65), or wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

The present invention further provides a method prevention and/or treatment of atherosclerosis in a subject, wherein said active derivative is lysophosphatidyl choline (LPC).

The present invention further provides a method prevention and/or treatment of atherosclerosis in a subject, wherein said LDL is malondialdehyde LDL (MDA-LDL).

The term "functional derivative" as used herein is intended to include labelled proteins, conjugated proteins, fused chimeric proteins and purified receptors in soluble form, as well as fragments, deletions, and conservative substitutions of said proteins.

The existence of an immune response against Ox LDL in atherosclerosis and the correlation between the reaction to Ox LDL and the severity of the disease, in combination with evidence that an active vaccine of Ox LDL in mice and rabbits can prevent the development of atherosclerosis has led the present inventors to conclude that the induction of immune tolerance by feeding Ox LDL to a human subject can result in the reduced rate of atherosclerosis progression. It should be mentioned that the mechanisms of inducing immune tolerance by mouth feeding are possibly mediated via a stimulation and production of cytokine TGFβ and the development of non-specific suppresser T-cells.

The oral tolerization of the present invention may extend to yield a bystandard suppression effect: namely - blocking other (non-antigen specific) autoimmune (anti-self) responses occurring in the vicinity of the atherosclerotic plaque and contributing to its progression.

It should be noted that the aim of the present invention is to induce tolerization or paralyze the immune response towards the HSP65, rather than to achieve mere elevation in the serum to assist protein unfolding.

Therefore, in one aspect the present invention combines oral tolerance, Ox LDL and atherosclerosis, which is a disease caused in part by immune factors. Ox LDL has been reported to induce an immune reaction in mice and rabbits (in contrast to inducing immune tolerance) of Ox LDL antigens and an improvement in the atherosclerosis condition. In these animal models Ox LDL has not been reported to have been experimented with mouth feeding and has never been suggested for oral tolerance.

The pharmaceutical compositions for oral administration according to the present invention are prepared by methods known per se and the administration thereof is by known methods of oral administration.

An amount effective to treat the disorder hereinbefore described depends on the usual factors such as the nature and severity thereof and the weight of the mammal.

For oral administration, it is preferred that the active ingredient be administered in the form of a unit-dose composition.

Such compositions are prepared by admixture and are suitably adapted for oral administration in the form of tablets, capsules, oral liquid preparations, powders, granules, etc.

Tablets and capsules for oral administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tabletting agents, lubricants, disintegrants, colorants, flavorings, and wetting agent. The tablets may be coated according to well known methods in the art.

Suitable fillers for use include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as sodium starch glycolate. Suitable lubricants include, for example, magnesium stearate. Suitale pharmaceutically acceptable wetting agents include sodium lauryl sulphate.

These solid oral compositions may be prepared by conventional methods of blending, filling or tabletting. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art.

Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or with another suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example, sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or

ethyl alcohol; preservatives, for example methyl or proply p-hydroxybenzoate or sorbic acid, and if desired conventional flavoring or coloring agents.

Oral formulations also include conventional sustained release formulations, such as tablets or granules having an enteric coating.

Description of Preferred Embodiments

While the invention will now be described in connection with certain preferred embodiments in the following examples so that aspects thereof may be more fully understood and appreciated, it is not intended to limit the invention to these particular embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included within the scope of the invention as defined by the appended claims. Thus, the following examples which include preferred embodiments will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of formulation procedures as well as of the principles and conceptual aspects of the invention.

MATERIALS AND METHODS

Animals.

Twelve weeks old LDL-RD mice (hybrids of between the C57BL/6J and 129Sv strains) are created by homologous recombination. The mice are purchased from The Jackson Laboratory (Bar-Harbor ME). The LDL-RD mice are used since they have high cholesterol levels on chow diet (which are similar to human values) and develop significant atherosclerosis only when fed a high fat diet. The LDL-RD mice are either fed with a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol) or an atherogenic diet (Harlan, Teklad Premier Laboratory Diets, Madison, WI) containing: 1.25% cholesterol, 7.5% casein and 0.5%(wt/) sodium-cholate. The mice are maintained on 12 hour-dark/12 hour light cycles and are allowed to access food and water *ad libitum*.

LDL isolation, oxidation and characterization

Blood for lipoprotein isolation is collected in EDTA (1mg/ml) after 12 hours of fasting. LDL (density=1.019-1.063 g/l) is isolated from the plasma after density adjustment with Kbr^-, by preparative ultracentrifugation at 50,000 rpm/min for 22

hours, using type 50 rotor. The LDL preparations are washed by ultracentrifugation, dialyzed against a pH 7.4, 0.15 mol/L EDTA, passed through Acrodisc filter (0.22 um pore size) to remove aggregates, and stored under nitrogen in the dark.

LDL oxidation is performed by incubation of pre-dialyzed LDL (1 mg of protein/ml in EDTA-free PBS) with copper sulfate (10μM) for 24 h at 37° C. Lipoprotein oxidation is confirmed by analysis of thiobarbituric acid-reactive substances (TBARS) which measures malondialdehyde (MDA) equivalents by the lipid peroxidation test and also by analysis of the conjugated diene content of the lipoprotein.

Determination of anti-Ox LDL antibody titers.

Preparation of native and copper-oxidized LDL is preformed. Ninety six well polystyrene plates (Nunc Maxisorp, Denmark) are coated with either Ox LDL, native LDL (at concentration of 5 ug/ml in PBS) or PBS overnight at 4^0C. After washing four times with PBS containing 0.05% Tween and 0.001% aprotinin (Sigma, USA) the plates are blocked with 2% bovine serum albumin (BSA) for 2 hours at room temperature. Serum fractions are diluted to 1:50 in PBS 0.05% Tween 0.2% BSA. After an additional overnight incubation the plates are washed and alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch laboratory Inc., USA) are added and diluted to 1:10,000 in PBS 0.05% Tween-0.2% BSA for 1 hour at room temperature. After extensive washing, 1 mg/ml *p*-nitrophenyl-phosphate (Sigma) in 50 mM carbonate buffer containing 1mM $MgCl_2$ pH 9.8 is added as a substrate. The reaction is stopped after 30 min by adding 1 M of NaOH. The color is read at a 405 nm wavelength in a Titertek ELISA reader (S.L.T Laboratory Instruments, Vienna, Austria) and results expressed as O.D. at 405 nm. The titer of anti-Ox LDL antibodies is calculated by subtracting the value obtained from binding to native LDL from the binding to Ox LDL.

Inhibition assays are performed to confirm the specificity of anti-Ox LDL antibodies and to check for a possible cross-reactivity with HSP-an important immunogen in atherosclerosis. The concentration of HSP-65-immunized mouse serum giving half of the maximal binding to Ox LDL are determined and different inhibitors (i.e. HSP-65, Ox LDL, bovine serum albumin) are applied in increasing concentrations.

Det ction of Anti-HSP-65 Antibodies.

Recombinant HSP-65 (1μg/ml) in phosphate buffered saline (PBS, pH 7.2) are coated onto flat bottom 96-well ELISA plates (Nunc Maxisorp, Denmark) by overnight incubation at 4°C. After washings with 0.02% PBS Tween and blocking with 1% BSA in PBS, sera are added in different dilutions (1:50, 1:100, 1:200, in PBS) and incubated for 1hr at room temperature. Peroxidase conjugated rabbit anti-mouse IgG (Dako Ltd, High Wycombe, UK) are added and incubated for 1 hr at room temperature followed by 4 washings with PBS/Tween. Finally, 100μl of citrate phosphate buffer (0.1 M, pH 4.2) containing 0.53 mg/ml of 2,2-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma) are added, and absorbance is measured after 30 min at 490 nm in a Titertek ELISA reader.

Detection of anti-phospholipid antibodies.

A modified ELISA to determine reactivity of the mouse and human antibodies to PL is perfrormed. Microtiter plates (Nunc, Maxisorp, Denmark) are coated either with an anionic PL [cardiolipin (CL), phosphatidylserine (PS), phosphatidylinositol (PI)] and phosphatidylcholine (PC), all from Sigma Chemicals Co. St. Louis, Mo, at a concentration of 50 ug/ml and dissolved in ethanol, except for PC (choloroform-methanol, 1: 3). Plates are dried under vacuum and blocked with TBS containing 0.5% gelatin. The plates are then washed three times with TBS and different concentrations of mouse preparations are added to the wells treated with human β2GPI (5ug/ml for 30 min) or 0.1% gelatin/TBS alone. The binding of the antibodies is detected with goat anti-mouse alkaline phosphatase conjugate and the addition of substrate (*p*-nitro-phenylphosphate). The results are expressed as absorbance at 405 nm (OD_{405}).

Detection of anti-b2GPI antibodies.

One ug/ml β2GPI or a different DM is incubated overnight in 50 mM bicarbonate buffer, pH 9.6 at 4^0C in 96-well polystyrene plates (Nunc). After 3 washings with TBS, blocking is performed with 0.5% gelatin/TBS for 2 hrs (as for the anti-PL ELISA). The plates are then washed three times and murine sera (diluted in 1: 100 in gelatin/TBS) is added and incubated for 2 hrs at room temperature. After 3 washings, alkaline phosphatase conjugated goat anti-human IgG respectively diluted in 0.1% gelatin/TBS (1:10,000) is added for 2 hrs. After 3

additional washings, the substrate *p*-nitrophenylphosphate in a sodium carbonate buffer pH 9.8 is added and absorbance is read at 405 nm.

Proliferation Assays of Spleen Lymphocytes.

Spleens are removed from mice upon sacrifice, and lymphocytes are isolated. $1x10^6$ cells per ml are incubated in triplicates for 72 h in 0.2 ml of culture medium in microtiter wells in the presence of different concentrations of HSP-65, Ox LDL, β2GPI or ovalbumin. Proliferation is measured by the incorporation of [^{3}H] thymidine into DNA during the final 12 hours of incubation. The results are computed as stimulation index (S.I.): the ratio of the mean cpm of the antigen to the mean background cpm obtained in the absence of the antigen.

Cytokine level determination

Spleen cells are removed following sacrifice and splenocytes are incubated for 3 days with Ox LDL, HSP-65 or ovalbumin following which the supernatant is collected . Cytokine profile (IL-4, IL-10, IFN-γ, and TGF-β) is determined at the cultured supernatant of the splenocytes.

Examples

Example 1

The mice used in this experiment are called LDL-receptor deficient (LDL-RD) mice. These animals have a genetic mutation which causes a defect in the receptor for LDL in all cells of the body. This receptor is responsible for evacuating and removing from the circulation, the 'bad' cholesterol (LDL), and when it is deficient the mice become hyperlipidemic and develop atherosclerosis when they are fed a high fat diet for 3-5 weeks.

Three groups of mice were used (15 LDL-RD mice in each group). Feeding was performed by a special device (canula) designed to be introduced to the esophagus and thus assuring the fed substances reaches mostly to the stomach.

Group 1: The mice were fed 5 doses of 1mg of human Ox LDL dissolved in PBS, every other day. At the end of the last dose, the mice were put on a high fat diet and sacrificed either after 3 or after 5 weeks of diet.

Group 2: The mice were fed 5 does of 1 mg of a control protein (ovalbumin) in PBS and then fed with the special diet for 3 or 5 weeks.

Group 3: The mice were not fed prior to initiation of the diet.

The mice were evaluated, upon sacrifice for the occurrence of atherosclerotic lesions, serum cholesterol values and levels of antibodies to Ox LDL.

Results: All mice were of similar weight prior to, and at the termination of the study. The mice fed OX LDL were found to develop less atherosclerosis (30% less).

In the present studies the beneficial effect of oral tolerance with Ox LDL on the induction of atherosclerosis in mice has been shown. There are many studies in which the results point to the effectiveness of oral tolerance in various autoimmune diseases, such as collagen in adjuvant arthritis (the analogue of rheumatoid arthritis), in diabetes mellitus, in uveitis, in EAE (analogue to multiple sclerosis). In the above-mentioned studies human trial was done. Recently a remarkable effect was noted when patients with rheumatoid arthritis were given per oral collagen II (oral tolerance). The comparable effect between mice studies and humans in various autoimmune diseases point to the extrapolated success in humans. It should be stressed that this treatment has no side effects.

Example 2

Suppression of high fat diet induced atherosclerosis in LDL-receptor deficient mice by feeding with human Ox LDL

LDL-RD mice (n=15) are fed 5 doses (every other day) of human Ox LDL in three different concentrations (1mg/dose, 100ug/dose and 1mg/dose). Additional LDL-RD mice (n=15 per groups) are fed a control antigen (ovalbumin at similar doses).

One day following the last feeding, all mice are challenged with a high fat diet ('paigen') for 5 weeks.

At the end of the experiment, sera from all mice is evaluated for the presence of anti-Ox LDL, anti-HSP65 and antiphospholipid antibodies and a lipid profile is carried out (total cholesterol, LDL, VLDL, HDL and triglycerides).

Hearts from all mice are removed at the end of the study and frozen in -70^{0}C until use (see Materials and methods) Immunohistochemical studies on the cryostat sections are performed using monoclonal antibodies to CD3, CD4, CD8, macrophages, smooth muscle cells and lymphocyte activation markers.

A similar protocol of feeding is carried out using LPC an active derivative of Ox LDL.

Example 3

Suppression of Mycobaceterium tuberculosis (MT) induced atherosclerosis by feeding with HSP-65.

The present inventors have observed that LDL-RD mice immunized once, with MT develop enhanced early atherosclerosis

LDL-RD mice (n=15) are fed 5 doses (every other day) of recombinant HSP65 in three different concentrations (100mg/dose, 10ug/dose and 1ug/dose). Additional LDL-RD mice (n=15 per groups) are fed a control antigen (ovalbumin at similar doses).

One day following the last feeding, all mice are challenged by an immunization with heat killed suspension of MT (10 mg/ml; 100ug/mouse) emulsified in incomplete Freund's adjuvant. The mice are sacrificed 12 weeks following the immunization with MT.

At the end of the experiment, sera from all mice is evaluated for the presence of anti-HSP65 antibodies, and a lipid profile is carried out (total cholesterol, LDL, VLDL, HDL and triglycerides).

Proliferative responses from draining lymph node cells are evaluated to HSP65 from HSP65 tolerized mice and from control fed mice.

Cytokine levels (IL-4, IFN-γ, IL-10 and TGF-β) are evaluated in the supernatant collected from the medium in which lymphocytes are induced in-vitro with HSP65.

Hearts from all mice are removed and frozen in -70^{0}C. To determine the extent of atherosclerosis cryostat sections are performed (5um per section). The relevant sections (from the aortic sinus area) are stained with the Oil-red O and the extent of atherosclerosis is determined by counting the area counted by a grid.

Immunohistochemical studies on the cryostat sections are performed using monoclonal antibodies to CD3, CD4, CD8, macrophages, smooth muscle cells and lymphocyte activation markers.

Example 4

Suppression of atherosclerosis by feeding with human b2GPI

The procedure is similar to the one employed for Ox LDL and HSP-65 and is based on the observation that immunization with β2GPI induces early atherogenesis.

Assessment of the extent of atherosclerosis.

Quantification of atherosclerotic fatty streak lesions are carried out by calculating the lesion size in the aortic sinus (with a few modifications). Briefly, the heart and upper section of the aorta are removed from the animals and the peripheral fat cleaned carefully. The upper section is embedded in OCT medium and frozen. Every other section (10μm thick) throughout the aortic sinus (400μm) is taken for analysis. The distal portion of the aortic sinus is recognized by the three valve cusps which are the junctions of the aorta to the heart. Sections are evaluated for fatty streak lesions after staining with oil-red O. Lesion areas per sections are counted using a grid by an observer unfamiliar with the tested specimen.

The extent of atherosclerosis is evaluated at the level of the aortic sinus. Processing and staining of the tissue is carried out. Lesion area is quantified by the modified method of Rubin EM, Kraus RM, Spangler EA & al. (Inhibition of early atherogenesis in transegenic mice by human apolipoprotein AI. Nature 1991; 353: 265-267).

Immunohistochemistry of atherosclerotic lesions.

mAbs: Rat monoclonal antibodies *H129.19 (L3T4)*-anti-mouse CD4+ and *S3-6.7 (Ly-2)*-anti-mouse CD8a are obtained from PharMingen (San Diego, CA, USA) ; *MCA 497 (F4/80)*-anti-mouse macrophages are obtained from Serotec (Oxford, UK).

Immunohistochemical staining for CD4, CD8 and macrophages are carried out on aortic sinus 5 μm thick frozen sections. The sections are fixed for 4 min. in methanol at -20^{0}C followed by 10 min. incubation with ethanol at -20^{0}C. The sections are then blocked with non-immune goat serum for 15 min. at room temperature followed by incubation with CAS blocking reagent for 30 min. at room temperature. Subsequently, the rat-monoclonal anti-mouse CD4/CD8 is added for 1 hr aroom . After washings, affinity purified biotinylated rabbit anti-rat IgG antibodies

are added for 30 min at RT. After washings, the slides are incubated with 0.3% H_2O_2, followed by additional rinses and incubation with streptavidin-peroxidase conjugate for 30 min at RT. After washings, the slides are developed with 3 amino-9-ethylcarbonasole (AEC) substrate (Dako) for 15 min. Sections are counterstained with hematoxylin. Spleen sections are used as a positive control. Staining in the absence of 1st or 2nd antibody are used as a negative control.

Adoptive trasfer experiments

T-cells (either CD4 or CD8 cells) will be isolated from the tolerized and non tolerized mice in each of the studies and transferred to LDL-RD littermates which will be challenged with an atherogenic diet for 6 weeks. At the end of the experiments the hearts will be taken for evaluation of atherosclerosis and immunohisotchemistry.

It will be evident to those skilled in the art that the invention is not limited to the details of the foregoing illustrative examples and that the present invention may be embodied in other specific forms without departing from the essential attributes thereof, and it is therefore desired that the present embodiments and examples be considered in all respects as illustrative and not restrictive, reference being made to the appended claims, rather than to the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

WHAT IS CLAIMED IS:

1. An immunological oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

2. An immunological oral tolerance-inducing composition for prevention and/or treatment of a heart attack, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

3. An immunological oral tolerance-inducing composition for prevention and/or treatment of angioplasty-restenosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

4. An immunological oral tolerance-inducing composition for prevention and/or treatment of stroke, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

5. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is a modified low-density lipoprotein.

6. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is oxidized low-density lipoprotein (Ox LDL).

7. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL).

8. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is heat shock protein 60/65 (HSP 60/65).

9. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of HSP60/65.

10. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

11. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of β_2GP-1.

12. An immunological oral tolerance-inducing composition according to claim 1, wherein said active derivative is lysophosphatidyl choline (LPC).

13. An immunological oral tolerance-inducing composition according to claim 1, wherein said LDL is malondialdehyde LDL (MDA-LDL).

14. A method for prevention and/or treatment of atherosclerosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

15. A method for prevention and/or treatment of a heart attack in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

16. A method for prevention and/or treatment of angioplasty-restenosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional

derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

17. A method for prevention and/or treatment of stroke in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

18. A method according to claim 14, wherein said active component is a modified low-density lipoprotein.

19. A method according to claim 14, wherein said active component is oxidized low-density lipoprotein (Ox LDL).

20. A method according to claim 14, wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL).

21. A method according to claim 14, wherein said active component is heat shock protein 60/65 (HSP 60/65).

22. A method according to claim 14, wherein said active component is an active derivative of heat shock protein 60/65 (HSP 60/65).

23. A method according to claim 14, wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

24. A method according to claim 14, wherein said active component is an active derivative of beta$_2$-glycoprotein-1 (β_2GP-1).

25. A method according to claim 14, wherein said active derivative is lysophosphatidyl choline (LPC).

26. A method according to claim 14, wherein said LDL is malondialdehyde LDL (MDA-LDL).

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 96/16007

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 621 341 A (AMERICAN CYANAMID CO) 26 October 1994 see page 3, line 15 - line 19 see page 3, line 48 - line 53 ---	1
X	IMMUNOLOGY TODAY, vol. 16, no. 8, 1995, CAMBRIDGE GB, pages 383-386, XP002034065 SYLVIE TREMBLEAU ET AL.: "THE ROLE OF IL-12 IN THE INDUCTION OF ORGAN-SPECIFIC AUTOIMMUNE DISEASES" see the whole document -----	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 1997	15.07.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Rempp, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 96/ 16007

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-14
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr al Application No
PCT/US 96/16007

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0621341 A	26-10-94	AU 6059194 A	27-10-94
		CA 2121096 A	23-10-94
		JP 7070198 A	14-03-95

INTERNATIONAL SEARCH REPORT

[redacted]onal Application No
PCT/IL 99/00519

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/17 A61P9/10 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal BIOSIS WPI MEDLIN CA EMBASE LIFESC SCISEA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GEORGE JACOB ET AL: "Induction of early atherosclerosis in LDL-receptor-deficient mice immunized with beta2-glycoprotein I." CIRCULATION SEPT. 15, 1998, vol. 98, no. 11, pages 1108-1115, XP000892137 ISSN: 0009-7322 the whole document ---	1-26
A	US 5 348 945 A (BERBERIAN P.A. ET AL.) 20 September 1994 (1994-09-20) cited in the application the whole document ---	1-26

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 2000	17/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 BONNIN DUSTAN ET AL: "Mucosal modulation of immune responses to heat shock proteins in autoimmune arthritis." Database accession no. PREV199800228934 XP002136641 abstract & BIOTHERAPY (DORDRECHT) 1998, vol. 10, no. 3, 1998, pages 213-221, ISSN: 0921-299X	1-26
P,X	GEORGE J ET AL: "Atherosclerosis-related markers in systemic lupus erythematosus patients: The role of humoral immunity in enhanced atherogenesis." LUPUS 1999, vol. 8, no. 3, 1999, pages 220-226, XP000906739 ISSN: 0961-2033 the whole document	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 99/00519

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 14-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied th payment of additional search fees.

INTERNATIONAL SEARCH REPORT

[redacted]tion on patent family members

[redacted]onal Application No
PCT/IL 99/00519

Patent document cited in search report	Publication date	Patent family member(s)	Publication dat
US 5348945 A	20-09-1994	AU 659085 B	11-05-1995
		AU 7677391 A	30-10-1991
		CA 2079813 A	07-10-1991
		EP 0527783 A	24-02-1993
		WO 9115219 A	17-10-1991

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
126,447 PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL99/00519	30/09/1999	04/10/1998
International Patent Classification (IPC) or national classification and IPC A61K38/17		
Applicant SHOENFELD, Yehuda et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/05/2000	10.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Deck, A Telephone No. +49 89 2399 8432

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL99/00519

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-9,14-22	as originally filed			
10-13	as received on	30/05/2000	with letter of	23/05/2000

Claims, No.:

1-6	as received on	30/05/2000	with letter of	23/05/2000
7-26	as received on	12/10/2000	with letter of	10/10/2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 14-26.

because:

   ☒ the said international application, or the said claims Nos. 14-26 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-26
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-26

	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-13
	No:	Claims	

2. Citations and explanations
see separate sheet

Concerning section III:

Claims 14 to 26 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Concerning section V:

The present application meets the requirements of Article 33 (2) and (3) PCT: the available prior art neither discloses nor suggests oral tolerance-inducing compositions for treatment of atherosclerosis, heart attack, angioplasty-restenosis or stroke comprising either LDL, Ox LDL, HSP 60/65, β_2GP-1 or functional derivatives thereof in combination with an oral carrier.

For the assessment of the present claims 14 to 26 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

U.S. Patent No. 5,348,945 discloses a method of combating mortality in a cell or tissue under stress. The method comprises contacting heat shock protein 70 (HSP70) to the cell or tissue in an amount effective to enhance the survival of that cell or tissue. The method may be employed in the combating of atherosclerosis, restenosis after angioplasty and nerve damage in human or animal subjects in need of such treatment. A pharmaceutical composition comprising a therapeutically effective amount of HSP70 in a pharmaceutically acceptable formulation is also disclosed.

Although HSP70 and HSP60 belong to a family of about 24 highly conserved heat shock proteins, they represent two entirely distinct characteristics. Their mechanism, for example, do not appear to act in concert in governing the protection from stressful stimuli.

HSP70 was initially patented because of its pronounced induction during heat exposure and other stressful insults such as ischemic preconditioning. Indeed the overexpression of HSP70 in transgenic animals is associated with protection from stressful hazards.

Therefore, U.S. Patent No. 5,348,945 does not teach or suggest the subject matter of the present invention.

Disclosure of the Invention

Thus, according to the present invention there is now provided an immunological oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In an preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of a heart attack, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In another preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of angioplasty-restenosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a further preferred embodiment of the present invention there is provided an immunological oral tolerance-inducing composition for prevention and/or treatment of stroke, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In even further preferred embodiments of the present invention there is provided an immunological oral tolerance-inducing composition wherein said active component is a modified low-density lipoprotein, or wherein said active component is oxidized low-density lipoprotein (Ox LDL), or wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL), or wherein said active component is heat shock protein 60/65 (HSP 60/65), or wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

The present invention provides an immunological oral tolerance-inducing composition, wherein said active derivative is lysophosphatidyl choline (LPC).

The present invention also provides an immunological oral tolerance-inducing composition, wherein said LDL is malondialdehyde LDL (MDA-LDL).

In another aspect of the present invention there is provided a method for prevention and/or treatment of atherosclerosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and

mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a preferred embodiment there is provided a method for prevention and/or treatment of a heart attack in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a further preferred embodiment there is provided a method for prevention and/or treatment of angioplasty-restenosis following angioplasty in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In an even further preferred embodiment there is provided a method for prevention and/or treatment of stroke in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In further embodiments of the present invention there is provided a method for prevention and/or treatment of atherosclerosis in a subject, wherein said active component is a modified low-density lipoprotein, or wherein said active component is oxidized low-density lipoprotein (Ox LDL), or wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL), or wherein said active component is heat shock protein 60/65 (HSP 60/65), or wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

The present invention further provides a method prevention and/or treatment of atherosclerosis in a subject, wherein said active derivative is lysophosphatidyl choline (LPC).

The present invention further provides a method prevention and/or treatment of atherosclerosis in a subject, wherein said LDL is malondialdehyde LDL (MDA-LDL).

The term "functional derivative" as used herein is intended to include labelled proteins, conjugated proteins, fused chimeric proteins and purified receptors in soluble form, as well as fragments, deletions, and conservative substitutions of said proteins.

The existence of an immune response against Ox LDL in atherosclerosis and the correlation between the reaction to Ox LDL and the severity of the disease, in combination with evidence that an active vaccine of Ox LDL in mice and rabbits can prevent the development of atherosclerosis has led the present inventors to conclude that the induction of immune tolerance by feeding Ox LDL to a human subject can result in the reduced rate of atherosclerosis progression. It should be mentioned that the mechanisms of inducing immune tolerance by mouth feeding are possibly mediated via a stimulation and production of cytokine TGFβ and the development of non-specific suppresser T-cells.

The oral tolerization of the present invention may extend to yield a bystandard suppression effect: namely - blocking other (non-antigen specific) autoimmune (anti-self) responses occurring in the vicinity of the atherosclerotic plaque and contributing to its progression.

It should be noted that the aim of the present invention is to induce tolerization or paralyze the immune response towards the HSP65, rather than to achieve mere elevation in the serum to assist protein unfolding.

Therefore, in one aspect the present invention combines oral tolerance, Ox LDL and atherosclerosis, which is a disease caused in part by immune factors. Ox LDL has been reported to induce an immune reaction in mice and rabbits (in contrast to inducing immune tolerance) of Ox LDL antigens and an improvement in the atherosclerosis condition. In these animal models Ox LDL has not been reported to have been experimented with mouth feeding and has never been suggested for oral tolerance.

WHAT IS CLAIMED IS:

1. An immunological oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

2. An immunological oral tolerance-inducing composition for prevention and/or treatment of a heart attack, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

3. An immunological oral tolerance-inducing composition for prevention and/or treatment of angioplasty-restenosis, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

4. An immunological oral tolerance-inducing composition for prevention and/or treatment of stroke, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

5. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is a modified low-density lipoprotein.

6. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is oxidized low-density lipoprotein (Ox LDL).

7. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL).

8. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is heat shock protein 60/65 (HSP 60/65).

9. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of HSP60/65.

10. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

11. An immunological oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of β_2GP-1.

12. An immunological oral tolerance-inducing composition according to claim 1, wherein said active derivative is lysophosphatidyl choline (LPC).

13. An immunological oral tolerance-inducing composition according to claim 1, wherein said LDL is malondialdehyde LDL (MDA-LDL).

14. A method for prevention and/or treatment of atherosclerosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

15. A method for prevention and/or treatment of a heart attack in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

16. A method for prevention and/or treatment of angioplasty-restenosis in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional

derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

17. A method for prevention and/or treatment of stroke in a subject, comprising administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

18. A method according to claim 14, wherein said active component is a modified low-density lipoprotein.

19. A method according to claim 14, wherein said active component is oxidized low-density lipoprotein (Ox LDL).

20. A method according to claim 14, wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL).

21. A method according to claim 14, wherein said active component is heat shock protein 60/65 (HSP 60/65).

22. A method according to claim 14, wherein said active component is an active derivative of heat shock protein 60/65 (HSP 60/65).

23. A method according to claim 14, wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

24. A method according to claim 14, wherein said active component is an active derivative of beta$_2$-glycoprotein-1 (β_2GP-1).

25. A method according to claim 14, wherein said active derivative is lysophosphatidyl choline (LPC).

26. A method according to claim 14, wherein said LDL is malondialdehyde LDL (MDA-LDL).

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

GOLLER, Gilbert
WOLFF BREGMAN AND GOLLER
P.O. Box 1352
Jerusalem 91013
ISRAEL

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	10.01.2001
Applicant's or agent's file reference: 126,447 PCT	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL99/00519	30/09/1999	04/10/1998

Applicant: SHOENFELD, Yehuda et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Hundt, D Tel.+49 89 2399-8042

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
126,447 PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL99/00519	30/09/1999	04/10/1998
International Patent Classification (IPC) or national classification and IPC A61K38/17		
Applicant SHOENFELD, Yehuda et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/05/2000	10.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Deck, A Telephone No. +49 89 2399 8432

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL99/00519

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-9,14-22	as originally filed			
10-13	as received on	30/05/2000	with letter of	23/05/2000

Claims, No.:

1-6	as received on	30/05/2000	with letter of	23/05/2000
7-26	as received on	12/10/2000	with letter of	10/10/2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 14-26.

because:

   ☒ the said international application, or the said claims Nos. 14-26 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-26
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-26

	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-13
	No:	Claims	

2. Citations and explanations
see separate sheet

Concerning section III:

Claims 14 to 26 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Concerning section V:

The present application meets the requirements of Article 33 (2) and (3) PCT: the available prior art neither discloses nor suggests oral tolerance-inducing compositions for treatment of atherosclerosis, heart attack, angioplasty-restenosis or stroke comprising either LDL, Ox LDL, HSP 60/65, β_2GP-1 or functional derivatives thereof in combination with an oral carrier.

For the assessment of the present claims 14 to 26 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

U.S. Patent No. 5,348,945 discloses a method of combating mortality in a cell or tissue under stress. The method comprises contacting heat shock protein 70 (HSP70) to the cell or tissue in an amount effective to enhance the survival of that cell or tissue. The method may be employed in the combating of atherosclerosis, restenosis after angioplasty and nerve damage in human or animal subjects in need of such treatment. A pharmaceutical composition comprising a therapeutically effective amount of HSP70 in a pharmaceutically acceptable formulation is also disclosed.

Although HSP70 and HSP60 belong to a family of about 24 highly conserved heat shock proteins, they represent two entirely distinct characteristics. Their mechanism, for example, do not appear to act in concert in governing the protection from stressful stimuli.

HSP70 was initially patented because of its pronounced induction during heat exposure and other stressful insults such as ischemic preconditioning. Indeed the overexpression of HSP70 in transgenic animals is associated with protection from stressful hazards.

Therefore, U.S. Patent No. 5,348,945 does not teach or suggest the subject matter of the present invention.

Disclosure of the Invention

Thus, according to the present invention there is now provided an immunological and oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis by the oral administration thereof, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In an preferred embodiment of the present invention there is provided an immunological and oral tolerance-inducing composition for prevention and/or treatment of a heart attack by the oral administration thereof, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In another preferred embodiment of the present invention there is provided an immunological and oral tolerance-inducing composition for prevention and/or treatment of angioplasty-restenosis by the oral administration thereof, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a further preferred embodiment of the present invention there is provided an immunological and oral tolerance-inducing composition for prevention and/or treatment of stroke by the oral administration thereof, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In even further preferred embodiments of the present invention there is provided an immunological and oral tolerance-inducing composition wherein said active component is a modified low-density lipoprotein, or wherein said active component is oxidized low-density lipoprotein (Ox LDL), or wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL), or wherein said active component is heat shock protein 60/65 (HSP 60/65), or wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

The present invention provides an immunological and oral tolerance-inducing composition, wherein said active derivative is lysophosphatidyl choline (LPC).

The present invention also provides an immunological and oral tolerance-inducing composition, wherein said LDL is malondialdehyde LDL (MDA-LDL).

In another aspect of the present invention there is provided a method for prevention and/or treatment of atherosclerosis in a subject, comprising orally administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and

mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a preferred embodiment there is provided a method for prevention and/or treatment of a heart attack in a subject, comprising orally administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In a further preferred embodiment there is provided a method for prevention and/or treatment of angioplasty-restenosis following angioplasty in a subject, comprising orally administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In an even further preferred embodiment there is provided a method for prevention and/or treatment of stroke in a subject, comprising orally administering an immunological oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

In further embodiments of the present invention there is provided a method for prevention and/or treatment of atherosclerosis in a subject, wherein said active component is a modified low-density lipoprotein, or wherein said active component is oxidized low-density lipoprotein (Ox LDL), or wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL), or wherein said active component is heat shock protein 60/65 (HSP 60/65), or wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

The present invention further provides a method prevention and/or treatment of atherosclerosis in a subject, wherein said active derivative is lysophosphatidyl choline (LPC).

The present invention further provides a method prevention and/or treatment of atherosclerosis in a subject, wherein said LDL is malondialdehyde LDL (MDA-LDL).

The term "functional derivative" as used herein is intended to include labelled proteins, conjugated proteins, fused chimeric proteins and purified receptors in soluble form, as well as fragments, deletions, and conservative substitutions of said proteins.

The existence of an immune response against Ox LDL in atherosclerosis and the correlation between the reaction to Ox LDL and the severity of the disease, in combination with evidence that an active vaccine of Ox LDL in mice and rabbits can prevent the development of atherosclerosis has led the present inventors to conclude that the induction of immune tolerance by feeding Ox LDL to a human subject can result in the reduced rate of atherosclerosis progression. It should be mentioned that the mechanisms of inducing immune tolerance by mouth feeding are possibly mediated via a stimulation and production of cytokine TGFβ and the development of non-specific suppresser T-cells.

The oral tolerization of the present invention may extend to yield a bystandard suppression effect: namely - blocking other (non-antigen specific) autoimmune (anti-self) responses occurring in the vicinity of the atherosclerotic plaque and contributing to its progression.

It should be noted that the aim of the present invention is to induce tolerization or paralyze the immune response towards the HSP65, rather than to achieve mere elevation in the serum to assist protein unfolding.

Therefore, in one aspect the present invention combines oral tolerance, Ox LDL and atherosclerosis, which is a disease caused in part by immune factors. Ox LDL has been reported to induce an immune reaction in mice and rabbits (in contrast to inducing immune tolerance) of Ox LDL antigens and an improvement in the atherosclerosis condition. In these animal models Ox LDL has not been reported to have been experimented with mouth feeding and has never been suggested for oral tolerance.

WHAT IS CLAIMED IS:

1. An immunological and oral tolerance-inducing composition for prevention and/or treatment of atherosclerosis by the oral administration thereof, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

2. An immunological and oral tolerance-inducing composition for prevention and/or treatment of a heart attack by the oral administration thereof, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

3. An immunological and oral tolerance-inducing composition for prevention and/or treatment of angioplasty-restenosis by the oral administration thereof, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

4. An immunological and oral tolerance-inducing composition for prevention and/or treatment of stroke by the oral administration thereof, comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

5. An immunological and oral tolerance-inducing composition according to claim 1, wherein said active component is a modified low-density lipoprotein.

6. An immunological and oral tolerance-inducing composition according to claim 1, wherein said active component is oxidized low-density lipoprotein (Ox LDL).

7. An immunological and oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL).

8. An immunological and oral tolerance-inducing composition according to claim 1, wherein said active component is heat shock protein 60/65 (HSP 60/65).

9. An immunological and oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of HSP60/65.

10. An immunological and oral tolerance-inducing composition according to claim 1, wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

11. An immunological and oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of β_2GP-1.

12. An immunological and oral tolerance-inducing composition according to claim 1, wherein said active component is an active derivative of LDL which active derivative is lysophosphatidyl choline (LPC).

13. An immunological and oral tolerance-inducing composition according to claim 1, wherein said LDL is malondialdehyde LDL (MDA-LDL).

14. A method for prevention and/or treatment of atherosclerosis in a subject, comprising orally administering an immunological and oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

15. A method for prevention and/or treatment of a heart attack in a subject, comprising orally administering an immunological and oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

16. A method for prevention and/or treatment of angioplasty-restenosis in a subj ct, comprising orally administering an immunological and oral tolerance-inducing composition comprising an active component selected from the

group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

17. A method for prevention and/or treatment of stroke in a subject, comprising orally administering an immunological and oral tolerance-inducing composition comprising an active component selected from the group consisting of modified low density lipoprotein, oxidized low density lipoprotein (Ox LDL), heat shock protein 60/65 (HSP 60/65), beta$_2$-glycoprotein-1 (β_2GP-1), functional derivatives thereof and mixtures thereof, in combination with a pharmaceutically acceptable carrier for oral administration.

18. A method according to claim 14, wherein said active component is a modified low-density lipoprotein.

19. A method according to claim 14, wherein said active component is oxidized low-density lipoprotein (Ox LDL).

20. A method according to claim 14, wherein said active component is an active derivative of oxidized low-density lipoprotein (Ox LDL).

21. A method according to claim 14, wherein said active component is heat shock protein 60/65 (HSP 60/65).

22. A method according to claim 14, wherein said active component is an active derivative of heat shock protein 60/65 (HSP 60/65).

23. A method according to claim 14, wherein said active component is beta$_2$-glycoprotein-1 (β_2GP-1).

24. A method according to claim 14, wherein said active component is an active derivative of beta$_2$-glycoprotein-1 (β_2GP-1).

25. A method according to claim 14, said active component is an active derivative of LDL which active derivative is lysophosphatidyl choline (LPC).

26. A method according to claim 14, wherein said LDL is malondialdehyde LDL (MDA-LDL).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
126,447 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IL 99/00519	30/09/1999	04/10/1998
Applicant		
SHOENFELD, Yehuda et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of th **drawings** to be published with the abstract is Figure No. --

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 99/00519

Box I Observations where certain claim were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 14-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 99/00519

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/17 A61P9/10 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal BIOSIS WPI MEDLIN CA EMBASE LIFESC SCISEA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GEORGE JACOB ET AL: "Induction of early atherosclerosis in LDL-receptor-deficient mice immunized with beta2-glycoprotein I." CIRCULATION SEPT. 15, 1998, vol. 98, no. 11, pages 1108-1115, XP000892137 ISSN: 0009-7322 the whole document ---	1-26
A	US 5 348 945 A (BERBERIAN P.A. ET AL.) 20 September 1994 (1994-09-20) cited in the application the whole document ---	1-26

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 2000	17/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONS[redacted]ED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1998 BONNIN DUSTAN ET AL: "Mucosal modulation of immune responses to heat shock proteins in autoimmune arthritis." Database accession no. PREV199800228934 XP002136641 abstract & BIOTHERAPY (DORDRECHT) 1998, vol. 10, no. 3, 1998, pages 213-221, ISSN: 0921-299X	1-26
P,X	GEORGE J ET AL: "Atherosclerosis-related markers in systemic lupus erythematosus patients: The role of humoral immunity in enhanced atherogenesis." LUPUS 1999, vol. 8, no. 3, 1999, pages 220-226, XP000906739 ISSN: 0961-2033 the whole document	1-26

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
T/IL 99/00519

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5348945 A	20-09-1994	AU 659085 B	11-05-1995
		AU 7677391 A	30-10-1991
		CA 2079813 A	07-10-1991
		EP 0527783 A	24-02-1993
		WO 9115219 A	17-10-1991

W20 IMMUNE AND INFECTIOUS FACTORS IN ATHEROGENESIS

4.W20.1 Cytomegalovirus and vasculopathy

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Cytomegalovirus is a species specific beta herpesvirus. In the immunocompetent host CMV infections are mostly subclinical while in the immunocompromised host clinical symptoms occur. After primary infection the virus persists in the host in a latent state. The vessel wall, especially the smooth muscle cell layer is a site of latency for this virus. For several years, there is increasing evidence that CMV can be involved in vascular pathology. Starting with the work of Melnick in 1983 several reports indicate that CMV infections are thought to be play a role in neointimal formation. More recently the work of Speir indicate that CMV infections are one of the possible factors playing a role in restenosis. It is also clear that CMV infections have an influence on the pathogenesis of transplant associated arteriosclerosis.

Using a rat model we studied the infections of vascular cells and the role of the virus on the development of vessel wall pathology. Using the balloon denudation model it was found that neointimal smooth muscle cells are permissive cells of viral replication. Infection of rats resulted in activation of endothelial cells leading to enhanced adhesion of leukocytes while in the transplanted graft viral infection resulted in an enhanced transplant associated arteriosclerosis.

From these data we conclude that the vessel wall is a preferent site of infection and that infection leads to activation of the vascular system which at the end might lead to the development of vascular disease.

4.W20.2 Autoimmunity in atherosclerosis

Göran K. Hansson, Sten Stemme, Gabrielle Paulsson, Xinghua Zhou, Martin Bruzelius, Giuseppina Caligiuri, Antonino Nicoletti, Allan Sirsjö, Dirk Wuttge, Zhong-qun Yan. *Center for Molecular Medicine, Karolinska Hospital, Stockholm, Sweden*

Atherosclerosis is accompanied by a local immune response in the plaque but its role in the pathogenesis of the disease is still unclear. Although one might expect that an (auto)immune response would be an aggravating factor, some of its consequences could be protective. Studies of human plaques and of lesion formation in apo E-0 mice show that CD4+ T cells and macrophages form an inflammatory infiltrate. Both cell types are activated and secrete proinflammatory cytokines. CD4+ cells respond immunospecifically to oxidized LDL, suggesting that oxidation induces antigenic epitopes on LDL and converts it to an autoantigen. In early phases of disease, T cell antigen receptors show restricted heterogeneity, suggestive of an autoimmune response by a limited set of CD4+ T cells to a macrophage-presented antigenic epitope. The pathophysiological consequences of this response are probably mediated largely via cytokine secretion. The type of effectors evoked may partly depend on the stage and severity of disease. One of the most important mediators of proinflammatory T cell responses is NO, which is produced in large amounts in the plaque through the cytokine-inducible pathway. This data emphasize the importance of inflammation and immune responses in the pathogenesis of atherosclerosis.

4.W20.3 Role of mast cells in atherosclerosis: From fatty streak to rupture

P.T. Kovanen. *Wihuri Research Institute, Helsinki, Finland*

Immunohistochemical observations on human ahterosclerotic lesions have revealed that the lesions contain mast cells. These blood-borne cells are filled with cytoplasmic secretory granules that contain histamine, heparin, and neutral proteases chymase and tryptase. When activated, mast cells degranulate, and in this way may influence both extracellular and cellular metabolism in their immediate environment. Animal studies have suggested that the heparin proteoglycan fraction of exocytosed mast cell granules bind LDL, carry the bound LDL into macrophages, and so induce their conversion into foam cells. This process is further stimulated when chymase of the granules proteolyzes the bound LDL, renders it unstable and triggers its fusion. Granule chymase also proteolyzes HDL and reduces the HDL-dependent efflux of cholesterol from the foam cells. Functions for intimal mast cells other than those related to lipid metabolism are emerging; infiltrates of activated mast cells were detected at the site of coronary atheromatous erosion or rupture in myocardial infarction. Both chymase and tryptase may degrade extracellular matrix of the cap of the atheromas, either directly, or indirectly by activating metalloproteinases secreted by other cells in the vulnerable regions. Coronary mast cells, as sources of potent proinflammatory cytokines such as TNF-α, may also lead production of certain metalloproteinases by macrophages in the lesions, and so further weaken the rupture-prone areas of atheromas. These findings point to the possibility of a completely novel plaque-disrupting factor, that is the release from mast cells of proteases and cytokines that ultimately lead to matrix degradation in the plaque. Finally, the products released from mast cells may also prevent thrombus formation; heparin by inhibiting platelet-collagen interaction, and chymase by degrading and inactivating thrombin. The results point to multiple functions of mast cells in human atherogenesis, some being atherogenic and others antiatherogenic. Thus, the net result of mast cell stimulation may vary depending on the various stages of atherogenesis.

4.W20.4 Anti-inflammatory cytokines in the human atherosclerotic plaque

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Pro-inflammatory cytokines have been shown to be expressed in human atherosclerotic plaques and may be involved in plaque instability. However, the inflammatory process is generally controlled by a balance between pro and anti-inflammatory cytokines, including interleukin (IL) 4, IL-10 and IL-13. These cytokines are the product of Th2 cells. Yet, IL-10 is also abundantly produced by macrophages, and has been reported in *in vitro* studies to inhibit metalloproteinases and to stimulate the biosynthesis of the tissue inhibitor of metalloproteinases (TIMP-1) in mononuclear phagocytes. In addition, IL-10 possesses anti-apoptotic activity. Therefore, we studied the expression of IL-10 and IL-13 by immunohistochemistry in human carotid atherosclerotic plaques. Expression of TIMP-1 and occurrence of apoptosis (TUNEL method) were analyzed in adjacent sections. IL-10 and IL-13 were detected in all atherosclerotic plaques with variable degree of expression, but were not expressed in the underlying normal arterial wall. IL-13 was only expressed in lymphocytes whereas IL-10 was detected in both macrophages and smooth muscle cells. TIMP-1 was expressed in the atherosclerotic plaques, but not in the underlying normal arterial wall. The distribution of TIMP-1 was similar to that of IL-10 in macrophages, whereas a significant inverse correlation between IL-10 expression and apoptosis was obtained. Anti-inflammatory cytokines, IL-10 and IL-13, are expressed in human atherosclerotic plaques. IL-10 may be responsible for TIMP-1 production by macrophages in atherosclerotic plaques and for prevention of apoptosis. Expression of these anti-inflammatory cytokines might be an important counter-regulatory mechanism favoring plaque stabilization.

4.W20.5 Autoimmunity to heat shock proteins in atherosclerosis

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Based on data obtained from animal experiments and investigations in man, we have conceived a new "immunological" hypothesis for the development of atherosclerosis. This concept proposes that the first stage of this disease is of an inflammatory-immunological type. Humoral and cellular immune reactions against certain types of stress proteins, the so called heat shock proteins (hsp) 65/60, seem to play an important initiating role in atherogenesis. Stress proteins are phylogenetically highly conserved. Thus, an over 50% homology on the DNA and protein level exists between hsp 65 of *M. tuberculosis*, hsp 60 of *E. coli* (GroEl) and the mammalian homologue hsp 60. Immune reactions against bacterial hsp 60 are essential to protect an organism from infection. This protection has, however, to be "paid for" with the risk of cross-reactions to autologous hsp 60. Hsp 60 and adhesion molecules are expressed by endothelial cells at those sites of the arterial vascular tree that are subject to major haemodynamic stress, e.g. the aortic arch and the branching of larger vessels. Preexisting hsp 65/60-reactive T cells and antibodies, respectively, are able to react with these stressed endothelial cells and mediate the initial lesion. Protracted influence of these risk factors, especially high blood pressure and chemically modified (e.g. oxidized) low density lipoproteins, then lead to the development of classical atherosclerotic lesions, ranging from fatty streaks to fully blown plaques. Immunization of normocholesterolemic rabbits with recombinant hsp 65 leads to mononuclear cell infiltration at these predelection sites that can progress to classical atherosclerotic lesions, including foam cells, if the animals in addition receive a cholesterol-rich diet.

point" in the linear correlation between lipid composition (CE/TG-ratio) and melting temperature. Such a discontinuity is a strong indicator for an internal phase separation. Further evidence for such a demixing behaviour comes from electron spin resonance (ESR) studies on spin-labelled CE- and TG-molecules, enzymatically incorporated in the core of LDL. As the CE-spin probes are motionally restricted below and mobile above the phase transition, the TG-spin probes exhibit an isotropic behaviour throughout the temperature range, suggesting a different polarity of the lipid environment. Our present results constitute a major step towards a better understanding of the precise architecture of LDL particle core at different lipid compositions.

Supported by the Fond zur Förderung der wissenschaftlichen Forschung in Österreich, (P11697-MED to R.P.).

3.P.136 Competition-studies with antioxidized LDL autoantibodies

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In recent years, substantial evidence indicated that lipoproteins modified in vivo by free radical-mediated oxidation or nonenzymatic glycation may contribute in normal subjects and in patients with arteriosclerosis as well. Contradictory data reported by different groups make it difficult to associate the presence or progression of arteriosclerotic lesions with the occurrence of autoantibodies. We tried to eludicate which type of antigen promotes the formation of antibodies recognizing oxidized or modified LDL. In a first step we were screening subjects in a population of Graz (about 200 subjects, clinically healthy +atherosclerotic patients) to find high autoantibody titers by using Cu2+ -oxidized LDL as antigen. As mentioned above, the antigen which causes the formation of those antibodies is not known and it can not be excluded that the antigen is an oxidized protein other than apoB. In a second step one individual sera with high antibody titer was examined by competitive ELISAs using a wide range of competitors. We examined LDL modified by various lipid peroxidation products, such as HNE or MDA, macrophage-modified and enzyme-modified (lipoxygenase)-LDL, oxidized HDL and erythrocytes and oxidized microsomes. With this individual serum we tested, oxidized LDL was the best competitor but other antigens like lipoxygenase-, macrophage or MDA-modified LDL and oxidized erythrocytes also competeted with the binding of the antibodies to the solid phase. In conclusion it seems that autoantibodies to oxidized LDL not predominantly recognize oxidized LDL but other modified LDL's and proteins too, are of variable cross-reactivity and have moderate-to-low affinity. The results suggest that once an antibody is generated against an adduct between a lipid oxidation product and the apoprotein, it could also react with similar adducts formed elsewhere in the body. It may be of potential clinical relevance to detect the presence of autoantibodies. This can be used as an indicator of the extent of atherosclerosis or degree of in vivo oxidation.

3.P.137 A delayed transient elevation of protein kinase C activity is associated with oxidized lipoprotein(a)-induced production of plasminogen activator inhibitor-1 in vascular endothelial cells

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Increased level of lipoprotein(a) [Lp(a)] is a strong risk factor for premature cardiovascular diseases. Previous studies by our group indicate that oxidization enhances Lp(a)-induced production of plasminogen activator inhibitor-1 (PAI-1) in human umbilical vein endothelial cells (HUVEC) (Ren et al. 1997 Atherosclerosis 128:1–0). The present study demonstrates that treatment with 10 μg/ml of native or oxidized Lp(a) induced transient increases in protein kinase C (PKC) activity in cell lysate of HUVEC 15 min and 5.5 h after the beginning of the treatment. PKC activity in HUVEC were down-regulated following >36 h of incubation with native or oxidized Lp(a). Oxidized Lp(a) induced a greater down-regulation of PKC activity compared to native Lp(a), that was associated with a more prominent increase in PAI-1 production. Addition of 1 μM calphostin C, a specific PKC inhibitor, at the beginning or 5 h after the beginning of the treatment, but not at later stage (9 or 16 h after the beginning of the treatment), effectively inhibited oxidized or native Lp(a)-induced increases in PKC activity and the generation of PAI-1 during 48 h. The findings indicate that a delayed transient elevation of PKC activity is associated with oxidized or native Lp(a)-induced PAI-1 production in vascular EC (supported by Manitoba Medical Service Foundation).

3.P.138 Modified lipoproteins generate autoantibodies and accumulate in the arterial lesions of athero-diabetic patients and hamsters

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Data from literature indicate modified lipoproteins as highly immunogenic generating autoantibodies. To determine whether the level of autoantibodies correlates with the extent of atherosclerosis, we characterized and compared lipoprotein fractions isolated from plasma of coronary heart disease patients (CHD-P) and of a group of normal donors (N). All CHD-P (even normocholesterolemic) were characterized by a cholesterol ratio in LDL/HDL >4, while all N had this ratio <4. LDL from diabetic CHD-P was partly glycated, and had increased peroxides concentration. The level of anti-VLDL and anti-LDL circulating autoantibodies was double in CHD-P vs. N, as determined by ELISA (using isolated lipoproteins as antigens and autologous plasma as source of antibodies). The anti-LDL autoantibodies level correlated well with LDL cholesterol and negatively with the age of patients. For immunohistochemical detection of modified LDL we used anti-LDL, anti- HNE-Lys, and anti-AGE antibodies, followed by corresponding second antibodies coupled to FITC. Antibodies were applied on consecutive cryosections of the aortic arch, coronary arteries and aortic valves of myocardial infarction deceased patients and athero-diabetic hamsters (induced by hyperlipemic diet and streptozotocin injection) at various time points of micro- and macruangiopathy. The immunodetected antigens were co-localized in focal deposits, at the shoulders and base of the atheroma, and in the smooth muscle cells (SMC) of the fibrous cap. Modified proteins (AGE, HNE-Lys) were present either diffuse extracellularly or associated with macrophage-derived foam cells and lipid-loaded SMC of the enlarged intima. They were also present in SMC of small arteries in the adventicia. These data attest: (a) the presence of glycated and oxidized LDL in the plasma of patients and experimental animals; (b) the existence of an elevated level of circulating autoantibodies against VLDL and LDL; (c) the presence of glycated or oxidized LDL in detectable amounts in the atheroma, and indicate that modified lipoproteins are immunoactive components in the atherosclerotic process.

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3.P.139 Atherogenicity of low density lipoprotein does not correlate with the degree of its oxidation

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We have recently demonstrated, that lipids, particularly cholesterol, covalently bound to apolipoprotein-B (apoB) is a stable marker of low density lipoprotein oxidation (LDL). The present study is an attempt to assess the relationship between the degree of LDL oxidation (evaluated by the content of apo-B-bound cholesterol) and the ability of LDL to induce cholesterol accumulation in cultured human aortic intima smooth muscle cells, i.e. atherogenicity. ApoB-bound cholesterol content of LDL oxidized by copper ions, AAPH and sodium hypochlorite, after which LDL become atherogenic, markedly exceeded (3- to 5-fold) the content of apo-B-bound cholesterol in freshly isolated atherogenic and non-atherogenic LDL. Moreover, highly oxidized aggregate-free LDL preparations displayed no atherogenic activity in cell culture. Thus, the ability to induce cholesterol accumulation in cells, i.e., the atherogenicity of *in vitro* oxidized LDL is a result of LDL aggregation, but not oxidation. We also studied the relationship between LDL aterogenicity and apoB-bound cholesterol content in freshly isolated LDL from healthy subjects and normo- and hypercholesterolemic patients with coronary atherosclerosis. The ability of human LDL to induce cholesterol accumulation in culture cells did not correlate with the degree of *in vivo* LDL oxidation ($r = 0.10$, $n = 117$). It is concluded that LDL atherogenicity does not depend on the intensity of lipid peroxidation in LDL particles.

3.P.140 Multiple modification of low density lipoprotein occurs in human plasma

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We have earlier found in the human blood a fraction of low density lipoprotein (LDL) which is characterized by a reduced content of sialic acid. Desialylated LDL also has a low neutral carbohydrate level, decreased content of

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ABSTRACT: The recent negative results of the 2-year phase III trial of enteral myelin (**Myloral**), a preparation of bovine myelin given orally in multiple sclerosis (MS), warn that the efficacy of oral tolerization (ie, inducing hyporesponsiveness to a specific autoantigen) and the attractiveness of oral desensitization in animal models of MS may not translate into clinical practice. However, such **failure** should not deter further examination of the mouth, an immunologically interesting and patient-friendly route for immunomodulatory proteins.

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Therapeutic approaches in multiple sclerosis: Lessons from failed and interrupted treatment trials
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The therapy for multiple sclerosis (MS) has changed dramatically over the past decade. Recent immunobiological findings and current pathophysiological concepts together with advances in biotechnology, improvements in **clinical** trial design and development of magnetic resonance imaging have led to a variety of evaluable therapeutic approaches in MS. However, in contrast to the successfully introduced and established immunomodulatory therapies (e.g. interferon-beta and glatiramer acetate), there have been a remarkable number of therapeutic failures as well. Despite convincing immunological concepts, impressive data from animal models and promising results from phase I/II studies, the drugs and strategies investigated showed no benefit or even turned out to have unexpectedly severe adverse effects. Although to date there is no uniformly accepted model for MS, there is agreement on the significance of inflammatory events mediated by autoreactive T cells in the CNS. These can be modified therapeutically at the individual steps of a hypothetical pathogenetic cascade. Crucial corners like: (i) the prevalence and peripheral activation of CNS-autoreactive T cells in the periphery; (ii) adhesion and penetration of T cells into the CNS; (iii) local activation and proliferation and; (iv) de- and remyelination processes can be targeted through their putative mediators. Like a 'specificity pyramid', therapeutic approaches therefore cover from general immunosuppression up to specific targeting of T-cell receptor peptide major histocompatibility (MHC) complex. We discuss in detail **clinical** MS trials that failed or were discontinued for other reasons. These trials include cytokine modulators [tumour necrosis factor (TNF)-alpha antagonists, interleukin-10, interleukin-4, transforming growth factor-beta2], immunosuppressive agents (roquinimex, gusperimus, sulfasalazine, cladribine), inducers of remyelination [intravenous immunoglobulins (IVIg)], antigen-derived therapies [**oral tolerance**, altered peptide ligands (APL), MHC-Peptide blockade], T cell and T-cell receptor directed therapies (T cell vaccination, T-cell receptor peptide vaccination), monoclonal antibodies against leucocyte differentiation molecules (anti-CD3, anti-CD4), and inactivation of circulating T cells (extracorporeal photopheresis). The main conclusions that can be drawn from these 'negative' experiences are as follows. Theoretically promising agents may paradoxically increase disease activity (lenercept, infliximab), be associated with unforeseen adverse effects (e.g. roquinimex) or short-term favourable trends may reverse with prolonged follow-up (e.g. sulfasalzine). One should not be too enthusiastic about successful trials in animal models (TNFalpha blockers; **oral tolerance**; remyelinating effect of IVIg) nor be irritated by non-scientific media hype (deoxyspergualine; bone marrow transplantation). More selectivity can imply less efficacy (APL, superselective interventions like T-cell receptor vaccination) and antigen-related therapies can stimulate rather than inhibit encephalitogenic cells. Failed strategies are of high importance for a critical revision of assumed immunopathological mechanisms, their neuroimaging correlates, and for future trial design. Since failed trials add to our growing understanding of multiple sclerosis, 'misses' are nearly

as important to the scientific process as the 'hits'.

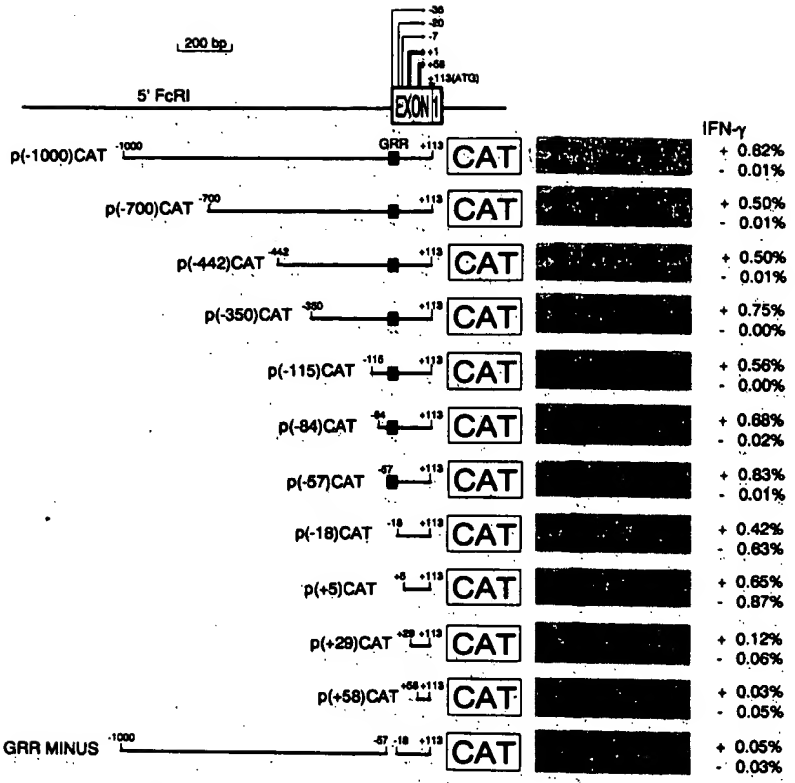

FIG. 4. Deletion analysis of the 5′ end of the Fc$_\gamma$RI promoter. Plasmids with progressive 5′ deletions of p(−1000)CAT were transfected into U-937 cells. Shown are the resultant CAT activities with percent conversion of [^{14}C]chloramphenicol. GRR is the 39-bp region from −57 to −18 defined by this analysis. GRR MINUS is p(−1000)CAT from which the GRR has been deleted.

exon to encode its transmembrane and cytoplasmic domains. Three cDNAs have been isolated for huFc$_\gamma$RI (30), two of which demonstrate allelic differences (p135 and p90), while the third (p98/X2) contains divergent 3′ sequence. The genomic sequence reported here is consistent with cDNA p135, with the following exceptions: at what would be cDNA nucleotide 1, a T, rather than a G, was found, and at amino acid 338 an isoleucine (coded by ATT) was found rather than a threonine (coded by ACT). Southern blot analysis of human DNA is consistent with the presence of two highly homologous genes in the genome (34). However, no evidence for a genomic copy of the divergent 3′ sequence has been found, either through screening genomic libraries with this sequence or by PCR amplification of RNA or DNA using this sequence as a primer (data not shown).

Within the promoter of the huFc$_\gamma$RI gene, we find that the 170 bp immediately upstream from the start of translation are sufficient to (*i*) drive transcription of a promoterless reporter

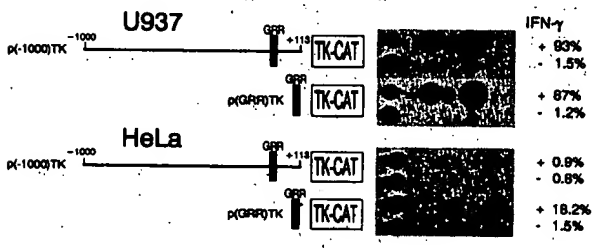

FIG. 5. The GRR is sufficient for IFN-γ induction of a heterologous promoter. Shown are CAT activities and percent conversion after transfection of p(GRR)TK and p(−1000)TK into U-937 and HeLa S3 cells. Conversion of [^{14}C]chloramphenicol after transfection of TKCAT into either U-937 or HeLa cells ranged between 1% and 2% and did not change with IFN-γ treatment.

gene in macrophage-like cell lines, (*ii*) mediate IFN-γ activation, and (*iii*) confer cell-type specificity. Further, we define the region responsible for IFN-γ induction as the 39 bp at the 5′ end of this 170-bp domain, from −57 to −18. We have shown that deletion of this 39-bp region, the GRR, from the huFc$_\gamma$RI promoter eliminates all induction by IFN-γ, and that a single copy of this GRR can confer strong IFN-γ inducibility to a heterologous reporter in both macrophage and non-macrophage cell lines.

Although the GRR acts as a transcriptional activator in the presence of IFN-γ, it appears to act as a negative regulator in its absence. This is suggested by the high constitutive activity seen after deletion of the GRR by exonuclease III (Fig. 4). This basal activity also implies the existence of a basal promoter for huFc$_\gamma$RI located 3′ to the GRR. Additional negative regulatory elements capable of suppressing this basal promoter appear to reside upstream of the GRR, as elimination of just the GRR from 1.1 kb of huFc$_\gamma$RI promoter does not result in constitutive activity.

The cell-type specificity exhibited by the huFc$_\gamma$RI gene may in part be a property of its basal promoter. This is suggested by the lack of activity in HeLa cells of all exonuclease III-generated mutants. Additional control of cell type-specific expression appears to reside in negative regulatory elements within the Fc$_\gamma$RI promoter, but outside the GRR. Alone, the GRR is not cell type-specific, as it can confer high IFN-γ inducibility upon a heterologous promoter in HeLa cells. However, in the context of 1.1 kb of Fc$_\gamma$RI promoter the GRR is inactive in HeLa cells.

Examinati n of the GRR reveals the presence of elements th ught t confer IFN-γ inducibility upon MHC class II promoters: an X box, an H box, and a γ-IRE (Fig. 6). The role of these elements within MHC class II promoters has been reviewed (17, 18). The X box has been found necessary for

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Lest anyone forget that before the Internet there were investment booms and busts in promising technologies, consider the tale of Howard Weiner, a Harvard neurologist and researcher who set out in the early 1980s to find a cure for multiple sclerosis. He didn't. But the story of his journey, which included the creation and collapse of his biotechnology company, AutoImmune Technologies, is a fascinating one, chronicled by Susan Quinn in Human Trials: Scientists, Investors and Patients in the Quest for a Cure.

Weiner believed that a cure for MS could be found in an oral treatment that addressed inflammation of the myelin, the covering around nerve cells in the brain and spinal cord that is attacked in MS. The proposed treatment was a dosage of myelin itself, which Weiner theorized would prompt the body to produce antibodies to fight the disease.

In 1986 Weiner and his team treated a group of mice with myelin protein and then injected them with the equivalent of MS for mice, The mice did not develop the disease. Two years later Weiner launched AutoImmune Technologies with $2.5 million from two venture capitalists, CW Group co-founders Walter Channing and Barry Weinberg, whom he had met through venture advisory panel.

Soon after, AutoImmune conducted a one-year clinical trial with 30 MS patients. Not even the subjects' doctors knew whether they were taking myelin or a placebo. The results were encouraging: Of the 15 people who took the oral protein, only six had an attack of MS, compared with 12 of the 15 who took the placebo.

Though these results were not statistically significant, Weiner and his team at AutoImmune were euphoric. They hired Hambrecht & Quist and Montgomery Securities, which in January 1993 raised $39 million in an IPO. The stock went public at 13 -- the top of its range -- and soon reached a high of 18.

The company quickly moved to a Phase III clinical trial in which 500 MS patients received treatment over a two-year period. These results were devastating, showing no difference between AutoImmune's drug, named **Myloral**, and the placebo.

AutoImmune's prospects, and its stock, went steadily downhill after the Phase III results were released in early 1997; eventually, the stock hit a low of 40 cents a share. The company still exists -- it traded at $2.75 last month -- and research on its patented ideas continues alongside clinical trials for diabetes prevention and treatment for rheumatoid arthritis.

The drug business "is sort of like prospecting," Fred Bader, AutoImmune's head of operations, told the author. "You dig a hole in the ground, and you don't see anything. You keep digging, and you may be two inches away from a vein of gold and walk away."

Although retail investors, as usual, suffered serious losses, AutoImmune's venture capitalists, who bought at 3 and sold at 16, made good money on the deal. Still, the saga left them leery of biotech investing. "To be in the venture capital business, you need to be optimistic," CW Group's Weinberg told Quinn. "But I think we were overly optimistic. The advent of these biotechnology tools (has not reduced) either the risk, the